Improvement in Speed and Reproducibility of Proteolytic Digestion Using a Novel Sample Preparation Technology

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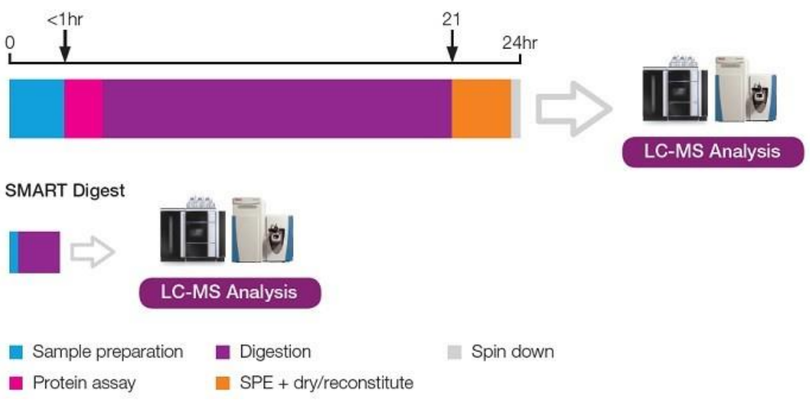
1 INTRODUCTION

The quantification of proteins by LC-tandem mass spectrometry (MS/MS) has become an important tool in the bioanalytical laboratory.

Historically, ligand binding assays (LBAs) have been the primary analytical technique to determine protein concentrations in bioanalytical samples. There are a number benefits afforded through the application of LC-MS/MS to the field of protein quantification. In comparison with LBA, LC-MS/MS benefits from enhanced selectivity, a larger dynamic range and greater accuracy. In addition to this LC-MS/MS can be readily multiplexed and can provide structural information without the need to generate costly critical reagents. Furthermore, as LC-MS/MS measures the protein molecule rather than a binding event, it is not reliant on reagent affinity and is not impacted by the presence of circulating target[1].

The most common LC-MS/MS bioanalytical methodologies for the quantification of proteins involves the detection of a specific surrogate peptide generated through a proteolytic digestion of biological samples: PrD-LC-MS/MS[2]. The most widely used enzyme for utilised in PrD-LC-MS/MS workflows is the endopeptidase trypsin. Trypsin is a serine protease that selectively hydrolyses at the COOH terminal of the amino acids lysine and arginine. Complete trypsinisation of a protein can take upwards of 16 hours and is a multi-stage process usually requiring reduction and alkylation of the analyte depending on the target peptide's position in the protein sequence and tertiary structure.

Figure 1: Savings Associated with SMART™ Digest



Thermo Scientific's SMART™ digest is a novel product comprised of immobilised trypsin on a solid support. The result is a thermally stable protease that allows for the use of temperature to accelerate the kinetics of the enzyme digestion. In addition to this the requirement to denature the protein is largely mitigated. This represents a significant saving in terms of time and cost (see Figure 1).

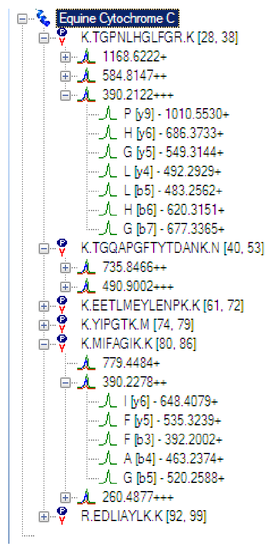
2 AIMS

- Assess the accuracy and precision achieved using a generic PrD-LC-MS/MS platform for routine laboratory use.
- Compare ease of utility of a SMART digest approach to that of conventional tryptic digest workflow.

3 MATERIALS AND METHODS

Cytochrome C (Cyt-C - Mw 11694.1) was selected as a test protein candidate owing to it's high digestion efficiency and low cost. Lactoglobulin was utilised as an internal standard owing to it's favourable chromatographic behaviour. Skyline ™ software was used to target signature peptides and predict their charge envelope prior to evaluation by MS/MS (see Figure 2).

Figure 2: Skyline *in-silico* protein digestion (Cyt-C)



Sequences observed in Q1 highlighted in red

Mass	Position	Sequence
547.27	2-6	GDVEK
634.39	10-14	IFVQK
1018.44	15-23	CAQCHTVEK
1168.62	29-39	TGPNLHGLFGR
1470.69	41-54	TGQAPGFTYTDANK
604.34	57-61	GITWK
1495.70	62-73	EETLMEYLENPK
678.38	75-80	YIPGTK
779.45	81-87	MIFAGIK
964.54	93-100	EDLIAYLK

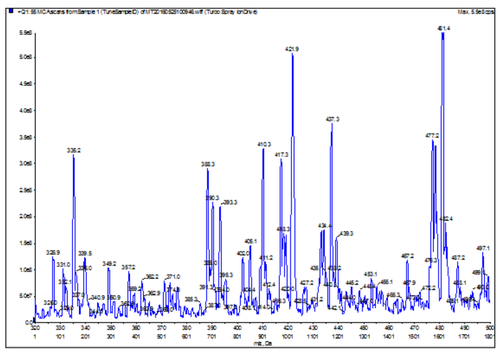
Approximately 70% sequence coverage

4 METHODOLOGY

MS/MS Optimisation

A Sciex API6500 MS/MS was used for this work. The most abundant Cyt-C precursor in the Q1 spectra corresponded to the TGPNLHGLFGR peptide and was visible in both 2+ and 3+ charge states (data not shown). This peptide was subsequently subjected to collision induced dissociation (CID) and product ions generated. The MS/MS was optimised to provide a multiple reaction monitoring (MRM) quantitation method for Cyt-C and lactoglobulin.

Figure 3: Example Q1 Scan (Cytochrome C)



Chromatography Development

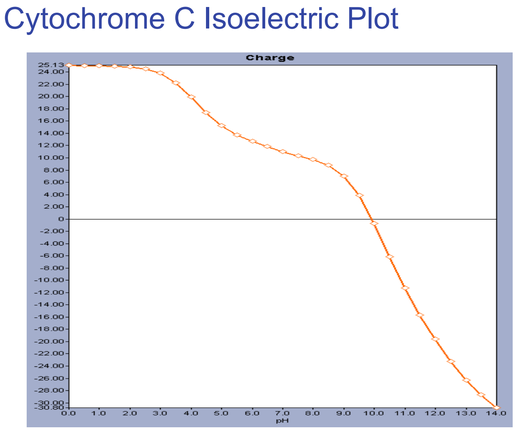
The chromatography was developed using an Agilent Poroshell EC-C18, 30 x 2.1 mm, 300 Å. Gradient elution was used running 2-30% acetonitrile/formic acid (100/0.1, v/v) over 2.2 mins. Example chromatography is shown in Figure 3.

Standard Digestion Protocol

Prior to digestion, the most abundant proteins were depleted using a reverse phase SPE (Thermo SOLA HRP). The isoelectric point (pI) of Cyt-C was estimated i*n-silico* at approximately pH 10. As albumin has a pI of ~4.7 it was possible to remove the most abundant protein(s) using a simple reverse phase SPE procedure.

0.1% w/v Rapigest™ SF was used to denature and solubilise the proteins to be digested. Reduction and alkylation with dithiothreitol and iodoacetamide was carried out prior to digestion with trypsin in 100 mM triethylammonium bicarbonate (TEAB). Samples were incubated at 40° C for 16 hours to facilitate digestion.

Figure 4: Cytochrome C Isoelectric Plot



SMART™ Digestion Protocol

50 µL of dog plasma was added to the SMART™ digest tube followed by 50 µL of digestion buffer. An Eppendorf Thermomixer C was used at 1400 rpm for 60 mins at 90° C The supernatant was directly injected. See figure 5 for an example LLOQ at 10 ng/mL achieved from 50 µL of plasma.

Figure 5: Example SMART Digestion Protocol

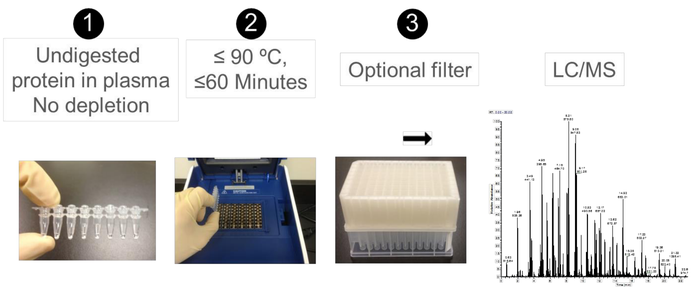
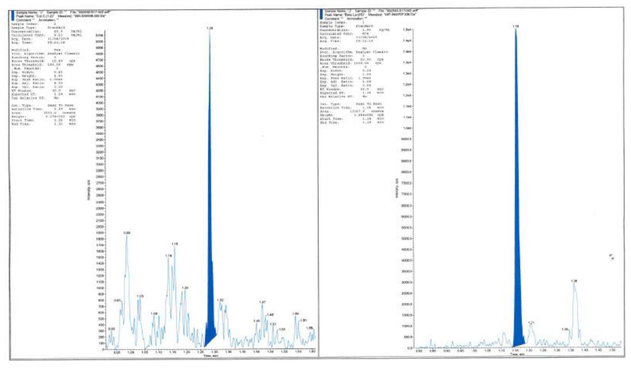


Figure 6: Example Extracted Chromatography



5 RESULTS

Accuracy and precision data achieved using conventional and SMART™ digests are shown in figures 7 and 8.

Figure 7: Conventional Digest Data

	Cytochrome C QC's (ng/mL)			
	LLOQ	Low	Medium	High
	10.0	25.0	500	8000
	8.89	28.8	557	8210
	-	-	504	7700
	-	32.8	526	8070
	11.0	24.3	498	8330
	11.0	36.8	570	8250
	10.6	19.6	518	8480
Mean	**10.4**	**28.5**	**529**	**8170**
CV %	**9.7**	**23.9**	**5.5**	**3.3**
Accuracy %	**103.7**	**113.8**	**105.8**	**102.1**

Figure 8: SMART™ Digest Data

	Cytochrome C QC's (ng/mL)			
	LLOQ	Low	Medium	High
	10.0	25.0	500	8000
	11.1	20.1	545	8230
	12.3	26.4	509	8280
	17.6	23.8	512	8750
	10.0	25.6	494	8530
	9.60	23.1	559	8840
	10.1	23.8	543	8740
Mean	**10.6**	**23.8**	**527**	**8560**
CV %	**10.2**	**9.2**	**4.8**	**3.0**
Accuracy %	**106.2**	**95.2**	**105.4**	**107.0**

6 CONCLUSION

- A method has been partially developed for the determination of Cyt-C using 50 µL of plasma.
- A conventional digestion protocol and intra-day data has been compared to SMART™ digestion
- SMART™ digestion reduces prep time from *ca* 24 hours to under 2 hours, improving ease of utility.
- SMART™ digestion demonstrated it's applicability for rapid and simple method development for Cyt-C.
- The developed procedure should be applicable to all protein targets. Assays for monoclonal antibodies are ongoing.

7 REFERENCES

[1] Bults P et al, Anal Chem (2016) Feb 2;88(3)1871-7

[2] Jenkins R, Duggan J. X. et al The AAPS Journal (2015) Vol. 17, No. 1